Voltage-dependent Na^+ channels in pyrethroid-resistant *Culex pipiens* L mosquitoes†

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Abstract: In some insect species, knockdown resistance (*kdr*) to pyrethroids and DDT is linked to point mutations in the sequence of the *para*-type voltage-dependent sodium channel gene. The effects of pyrethroids were assayed on six *Culex pipiens* strains: two were susceptible to pyrethroids and the four others displayed various levels of resistance, but, in each case, a *kdr*-type mechanism was strongly suggested. Degenerate primers were designed on the basis of the corresponding sequences of the *para* orthologous gene reported from several orders of insects. These primers were used to amplify the region of the sodium channel gene which includes the positions where the *kdr* and *super-kdr* mutations have been found in *Musca domestica*. As expected, the amplified fragment was highly homologous to the *para* sequences. The *super-kdr*-like mutation (methionine to threonine at position 918 of the *M domestica para* sequence) was never detected in any strain. In contrast, the same *kdr* mutation (leucine to phenylalanine at position 1014) was present in some *Culex* pyrethroid-resistant samples. An alternative substitution of the same leucine to a serine was detected in one strain slightly resistant to pyrethroids but highly resistant to DDT. These data have allowed us to design a PCR-based diagnostic test on genomic DNA to determine the presence or the absence of the *kdr* allele in single *C pipiens* collected in several countries. The validity of this test was checked by comparing the frequency of the resistance allele and the toxicological data.

Keywords: pyrethroid; resistance; knockdown; mosquito

1 INTRODUCTION

An important mechanism that confers resistance to pyrethroids and DDT, known as knockdown resistance or *kdr*, was first described in *Musca domestica* L[1] and has since been reported in many insect species.[2] It is best characterised by a reduced sensitivity of the insect nervous system to these compounds. The primary target site for pyrethroids and DDT is the voltage-dependent sodium channel of nerve-cell membranes[3] where the insecticides prolong the opening time of the channels.[4] Binding studies indicate a reduced affinity of sodium channels for pyrethroids in *kdr* flies.[5,6] Genetic mapping, which showed a tight linkage between *kdr* resistance and a sodium channel gene, further supported this conclusion.[7–10] Williamson *et al*[11] reported that a single mutation (leucine to phenylalanine) in the domain II of the sodium channel was the molecular basis of *kdr* in *M domestica*. An additional mutation (methionine to threonine) in the same region was responsible for the enhanced resistance to type II pyrethroids in *super-kdr* houseflies. Since then, the same *kdr* mutation (Leu to Phe) has been reported in various insect species including the German cockroach *Blatella germanica* (L)[12,13] the mosquito *Anopheles gambiae* Giles,[14] the lepidopteran *Plutella xylostella* (L)[15] and the aphid *Myzus persicae* Sulz.[16] A mutation affecting the same leucine residue has also been described in *Heliothis virescens*[17] although in this case a histidine instead of a phenylalanine was present in resistant insects. The effects of some of these mutations have been confirmed by in-vitro expression of mutated channels in *Xenopus* oocytes.[18,19]

The aim of this work was to find out whether the

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Table 1. Sequences of the primers used throughout this study

D1	5′ AARYTNGCNAARTCNTGGCC 3′
D2	5′ GCNAARTCNTGGCCNAC 3′
D4	5′ ACRAARTCNARCCARCACCA 3′
Dg2	5′ GCDATYTTRTTNGTNTCRTTRTC 3′
Dipd1	5′ TGGCCSACRCTKAAYTTACTC 3′
Dip2	5′ TTGGACAAAAGCAARGCTAAG 3′
Dip3	5′ ATCATCTTCATCTTTGC 3′
Dip4	5′ CATGCAGTCCCACATGG 3′
Cgd1	5′ GTGGAACTTCACCGACTTC 3′
Cgd2	5′ GCAAGGCTAAGAAAAGGTTAAG 3′
Cgd3	5′ CCACCGTAGTGATAGGAAATTTA 3′
Cgd4	5′ CCACCGTAGTGATAGGAAATTTT 3′

same mechanism is responsible for pyrethroid resistance in the mosquito *Culex pipiens* L, a major nuisance throughout the world and a vector of Bancroft filiarsiasis in tropical regions. We focused our attention on the analysis of the IIS4–IIS6 region of the sodium channel gene, since it contains the two amino acid positions responsible for *kdr* and *super-kdr* resistance, analysing pyrethroid-susceptible and pyrethroid-resistant strains from various geographical origins, including Mediterranean countries, Africa, North America and China.

2 MATERIALS AND METHODS

2.1 Strains

The two strains of *C pipiens quinquefasciatus* Say used in this study come from the USA: S-LAB is a standard susceptible strain,[20] and PERM was selected with α-*trans*-permethrin until homozygosity.[21]

Four strains of *C p pipiens* were used: BICHON which derives from the CYPRUS strain,[22] ESPRO and GARA isolated from collections made in Tunisia,[23,24] and CHANG deriving from a collection made in Guangzhou, southern China.[25] Strains were reared under standard laboratory conditions except for PERM which was selected with 0.1 mg litre^{-1} of α-*trans*-permethrin every five to six generations.

2.2 Bioassays

Assays were performed as previously described,[26] using alcohol solutions of permethrin (99%), (Riedel de Haen, Seelze, Germany), and DDT (99%), (Riedel de Haen). Most bioassays included five doses and four replicates per dose on sets of 20 early 4th instars in a total volume of 100 ml of water containing 1 ml of alcohol solution of the tested insecticide. The effects on resistance of three synergists (DEF = *S,S,S*-tributylphosphorotrithioate, Interchim, Montlucon, France, PB = piperonyl butoxide, Fluka AG, Buchs, Switzerland, and DMC = 1,1-di-[*p*-chlorophenyl] ethan-1-ol, Sherwin-Williams, Oakland, CA) were investigated by exposing larvae to a standard sublethal dose of 0.08 mg litre^{-1} for DEF, 5 mg litre^{-1} for PB, and 2 mg litre^{-1} for DMC, 4 h before adding the insecticide. Mortality was recorded after 24 h exposures and analysed using a log-probit program[27] based on Finney.[28] Resistance (RR) and synergism (SR) ratios were computed by choosing the option resistance ratio for non-parallel lines and they were considered significant ($P < 0.05$) if their 95% CI did not include the value 1.

2.3 RNA extraction, PCR, cloning and sequencing

The sequences of the primers used in this study are listed in Table 1. Total RNA was extracted from one to three insects using the guanidinium thiocyanate/phenol-chloroform method.[29] RNA (1–2 μg) was reversibly transcribed into single-stranded cDNA using the Superscript II reverse transcriptase (Life Technologies, France). The reaction was primed with a mixture of 200 ng of oligo-dT (Life Technologies, France) and 200 ng of an antisense sodium channel primer (D4) corresponding to a conserved sequence downstream of the IIS4–IIS6 region. Two rounds of PCR were carried out to selectively amplify the region of interest. The primary PCR reaction contained 1 unit of Taq DNA polymerase (Eurogentec, Belgium), 3 μl of cDNA, 250 ng of primers D1 and Dg2, 0.2 mM dNTPs, and the buffer provided by the polymerase supplier in a final volume of 50 μl. The reaction comprised 35 cycles of 94 °C for 1 min, 50 °C for 2 min and 72 °C for 2 min with a final extension step of 72 °C for 10 min. A secondary PCR was carried out in 100 μl with 1 μl of the primary reaction as template and primer D2 instead of D1, all other components being kept unchanged. The reaction profile was identical except that the annealing and extension steps were 1 min each. PCR products were separated on a 1.5% agarose gel and were visualised by ethidium bromide staining. The discrete 420 bp amplified fragments from the secondary reaction were excised from the gel and cloned into a T-tailed plasmid vector (Invitrogen, The Netherlands). Recombinant plasmids were sequenced on an Applied Biosystems 373 automated sequencer. The sequences were processed and analysed using Staden and Wisconsin GCG software packages.

2.4 Direct sequencing of PCR products

Primary PCR reactions were done on cDNAs as described above. The secondary PCR reactions were carried out using primers Dipd1 and Dip2 instead of D2 and Dg2 respectively, and the annealing temperature was reduced to 48 °C. The 359 bp amplified fragment was recovered by ammonium acetate-ethanol precipitation and used as template for direct sequencing using primers Dip3 and Dip4.

2.5 *kdr* diagnostic test

Two PCR reactions were run in parallel. In each of them, the template was 10 to 50 ng of genomic DNA prepared as reported earlier.[14] In one reaction, the primers Cgd1, Cgd2 and Cgd3 were combined and, in the other one, Cgd3 was replaced by Cgd4. The PCR conditions were 1 min at 94 °C, 2 min at 48 °C and

Table 2. Resistance characteristics of the *Culex pipiens* strains

Strain	*Synergist*	*LC_{50} (mg litre^{-1})*	*Slope*[a]	*RR_{50}*[b]	*SR_{50}*[c]
Permethrin					
S-LAB	none	0.0024 (0.0019–0.0034)	2.96 (0.52)	–	–
	PB	0.00019 (0.00015–0.00025)	4.49 (0.84)*	–	12 (6.9–22)
	DEF	0.0010 (0.00089–0.0012)	3.58 (0.51)	–	
PERM	None	2.56 (2.28–2.87)	2.28 (0.13)	1089 (845–1403)	–
	PB[e]	0.54	1.7	1286	16
	DEF[e]	4.9	1.6	7208	1.8
ESPRO	None	0.010 (0.0070–0.015)	2.01 (0.37)*	4.3 (2.9–6.4)	–
	PB	0.0010 (0.00088–0.0012)	1.52 (0.09)	5.2 (3.3)	0.61 (0.37–0.98)
	DEF	0.017 (0.010–0.028)	2.37 (0.67)*	17 (11–26)	0.61 (0.38–0.98)
CHANG	None	0.013 (0.012–0.014)	3.40 (0.35)	5.5 (4.2–7.1)	–
	PB	0.00087 (0.00076–0.00098)	2.18 (0.14)	4.5 (2.9–7.0)	15 (13–17)
	DEF	0.0073 (0.0051–0.011)	3.30 (1.54)*	7.2 (4.3–12)	1.7 (1.1–2.8)
GARA	None	0.14 (0.062–0.31)	0.79 (0.16)* ϕ	60 (43–84)	
	PB	0.00034 (0.00012–0.00083)	0.84 (0.17)* ϕ	1.7 (1.0–2.9)	419 (299–588)
	DEF	0.10 (0.072–0.15)	1.2 (0.11)* ϕ	102 (81–130)	1.3 (1.1–1.7)
Deltamethrin					
S-LAB	None	0.00012 (0.00011–0.00013)	5.36 (0.56)	–	–
PERM[d]	None	0.017	–	142	–
CHANG	None	0.0067 (0.00044–0.0010)	2.14 (0.88)*	5.6 (3.6–8.8)	–
DDT					
S-LAB	None	0.013 (0.012–0.015)	5.96 (0.62)	–	–
	DMC	0.018 (0.016–0.019)	5.10 (0.39)	–	0.73 (0.58–0.92)
PERM[d]	None	95	–	1610	–
CHANG	None	4.5 (3.7–5.5)	2.74 (0.41)*	350 (259–473)	–
	DMC	20 (5.7–88)	0.75 (0.19)*	1174 (812–1700)	0.22 (0.15–0.32)
GARA	None	0.035 (0.012–0.099)	0.60 (0.11)* ϕ	1.98 (1.5–2.5)[e]	–
	DMC	0.074 (0.050–0.11)	4.42 (1.97)*	2.7 (2.0–3.7)	1.9 (1.09–3.2)

[a] Standard deviation in parenthesis; *indicates that the linearity of dose-mortality response is rejected; ϕ indicates the presence of a clear plateau over more than one log scale so that RR at LC_{95} was much higher than at LC_{50}. At LC_{95}, GARA had a RR of 1977 (891–4383) with permethrin, of 497 (331–747) with permethrin +PB, and a DDT resistance of 70 (35–137).

[b] Resistance ratio = LC_{50} of the tested strain/LC_{50} of S-LAB.

[c] Synergism ratio = LC_{50} in presence of synergism/LC_{50} in absence of synergism; data from Priester and Georghiou:

[d] (1980);[31]

[e] (1978).[21]

2 min at 72 °C for 40 cycles. DNA fragments were separated by electrophoresis on 1.5% agarose gels and were visualised by ethidium bromide staining under UV light.

3 RESULTS AND DISCUSSION

3.1 Pyrethroid resistance in the various strains

Resistance characteristics of the strains studied are given in Table 2, and some mortality dose-responses are shown in Figure 1. BICHON, which has never been exposed to pyrethroid, was susceptible, whereas all other strains displayed a significant resistance (the confidence interval of their resistance ratio, RR, did not include the value 1).

The most resistant strain was PERM, which, as expected from previous studies, was highly resistant to permethrin (>1 000-fold). This strain has been thoroughly studied by Georghiou and his collaborators[21,30–32] who concluded that its resistance was due to a single major mutation affecting the pyrethroid target, ie the voltage-dependent Na^+ channel. This conclusion was based on the following observations: (a) synergists had a limited action on resistance,[21] (b) penetration was not higher than in susceptible insects,[30] (c) cross-resistance to DDT was high (1,600-fold),[31] (d) larvae remained unaffected by 24-h exposure to 1 mg litre^{-1} of permethrin whereas susceptible larvae were knocked down after a 10- to 20-min exposure to 0.01 mg litre^{-1},[30] and (e) permethrin resistance was due to a single major gene.[32]

ESPRO displayed a 4.3-fold resistance to permethrin and linearity of the dose-mortality response was rejected, mostly due to an inflection of the mortality curve at the highest doses (Fig 1A). Permethrin action was not synergised by DEF (an inhibitor of esterases) or by PB (an inhibitor of cytochrome P450 oxidases).

The GARA strain displayed a high resistance to permethrin. The response was highly heterogeneous with a plateau over two log-scales at a mortality between 70 and 80% (Fig 1A). The observed permethrin resistance ratio (RR) was 60 at LC_{50} and 1,977 at LC_{95}. Permethrin action was not synergised by DEF, indicating that no esterase was involved. In contrast, it was synergised by PB: the mortality plateau remained clearly visible and the whole mortality curve

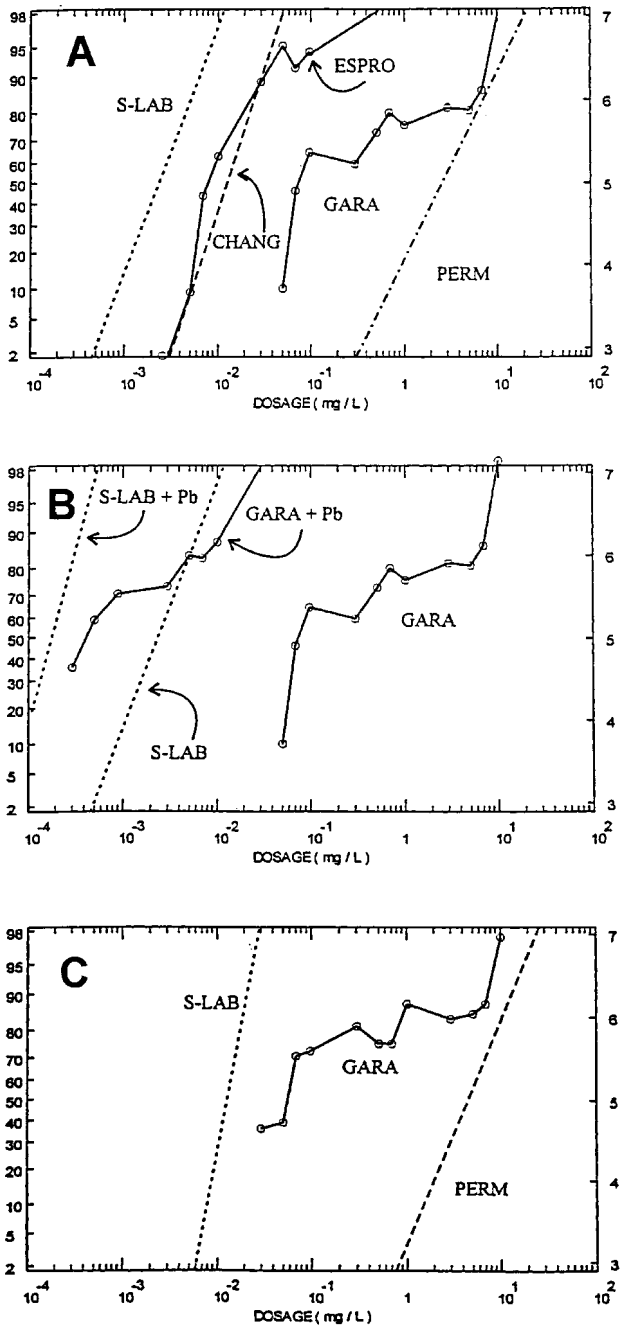

Figure 1. Dose-mortality responses observed in different *Culex pipiens* strains. A: Comparison of permethrin resistance in the susceptible reference strain S-LAB, in the Tunisian ESPRO and GARA strains, in the American PERM strain, and in the Chinese CHANG strain. B: Comparison of permethrin resistance in S-LAB and GARA strains when using permethrin alone or permethrin and piperonyl butoxide (PB). C: DDT resistance in S-LAB, GARA and PERM strains. In each figure, data are given for strains where the linearity of the dose-mortality response is rejected ($P < 0.05$).

appeared to be shifted towards lower doses (Fig 1B). The much higher PB synergism ratio in GARA (SR$\approx$419 at LC_{50}) than in S-LAB (SR = 12) indicated that cytochrome P450 oxidases were involved in the permethrin resistance of GARA. However, resistance at LC_{95} remained high (RR$\approx$34), indicating the presence of a non-detoxifying mechanism. Linearity of the DDT dose-mortality response of GARA larvae was also rejected and a plateau was observed at 70 to 80% mortality as with permethrin (Fig 1C). RR of GARA was estimated to be$\approx$2.0 at LC_{50} and$\approx$70 at LC_{95}. This resistance was not affected by DMC, an inhibitor of dehydrochlorinase (Table 2).

Permethrin, deltamethrin and DDT dose-mortality responses were linear in the CHANG strain, which showed an$\approx$5-fold resistance to both pyrethroids and a 350-fold resistance to DDT as compared to S-LAB (Table 2). Resistance to permethrin was not synergised by DEF or PB, and resistance to DDT was not synergised by DMC. These data indicate that pyrethroid and DDT resistances in CHANG are likely to be due to a modification of the target of these insecticides, ie a *kdr*-like mechanism.

In conclusion, toxicology data indicate that, if any modifications in the sodium channel gene are involved in the pyrethroid resistance observed for the CHANG and PERM strains, they are probably different. This modification in CHANG provides a low resistance to pyrethroids ($\approx$5-fold) and a high resistance to DDT (350-fold), whereas the modification in PERM is responsible for a high resistance to both pyrethroids and DDT (>1 000-fold, this study and Priester and Georghiou[31]). In addition, our results on the GARA strain show that the same mutation as in PERM is likely to be present, since high resistance to both permethrin and DDT was observed at LC_{95}. Finally, taking into consideration that ESPRO and GARA strains both derive from collections made in Tunisia, their permethrin resistance is probably due to the same sodium channel mutation. The mortality curve of ESPRO is consistent with the hypothesis that this mutation is at a very low frequency and that, when present, it is mostly in a heterozygous state (Halliday and Georghiou[32] have shown that *kd* resistance of PERM $\times$ S-LAB is$\approx$10-fold, ie almost recessive).

3.2 Alternative splicing of the *para* ortholog in *Culex*

The strategy for sequencing the domain IIS4–IIS6 region of the sodium channel gene homologous to the *Drosophila melanogaster para* gene relies on the use of degenerate primers whose nucleotide sequences are based on conserved amino-acid motives flanking the region of interest in vertebrates and invertebrates.[11,33] The high degeneracy of these primers prevents their use for direct sequencing of the amplified products, and cloning of even a very limited number of sequences is therefore necessary to design a set of more specific primers that can be used both to specifically amplify the sequences of interest and to perform direct sequencing of the amplified fragments.

By using this strategy, a fragment of about 420 pb was first amplified from S-LAB and PERM. This fragment was cloned into a T-tailed vector and inserts from five recombinant plasmids (two corresponding to the resistant strain and three to the susceptible one) were fully sequenced. The high similarity (82%) found between these sequences and the *Drosophila para*

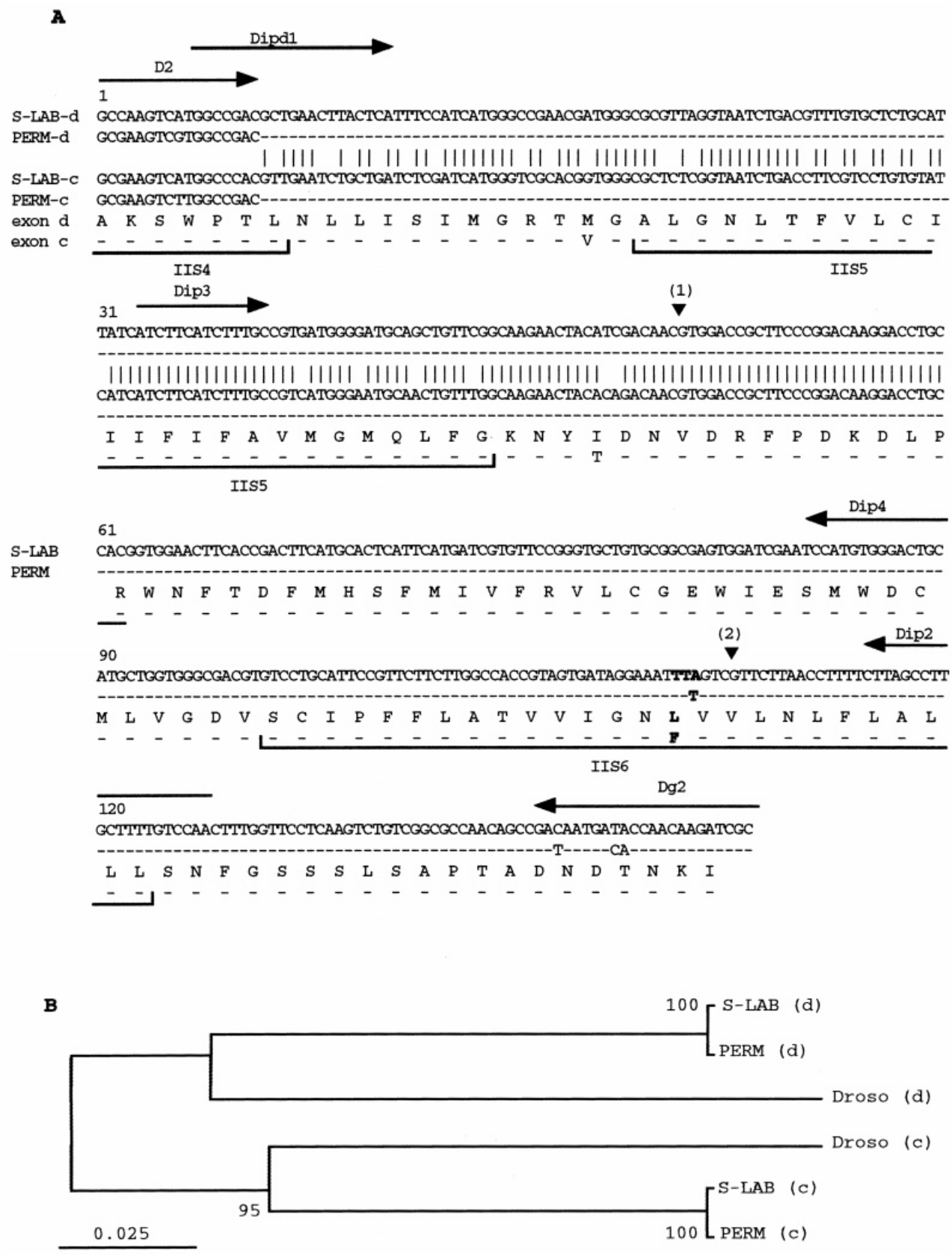

Figure 2. A: Alignment of the nucleotide sequences obtained from cloned products for the strains S-LAB and PERM. Sequences of the two alternative exons (c and d) are included. Identical positions are indicated by (–). Identities between the two alternative exons are indicated by (|). Below the nucleotide sequences are the deduced amino acid sequences. The triplet involved in the Leu–Phe mutation is in bold face. ▼ indicates the positions of the two introns present in *Drosophila melanogaster* in this region. Arrows indicate the positions of the various primers. Position 1 corresponds to position 905 in the housefly amino acid sequence.[11] The sequence has been deposited in the GenBank (accession number AJ012476). B: A Neighbour Joining Tree based on the Jukes and Cantor distances estimated for the nucleotide sequences showing the orthologous relationships between the two alternative exons in *Culex* and *Drosophila*. Numbers at the nodes indicate percentage bootstrap values supporting each particular node based on 1000 replicates.

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                909
Musca           PTLNLLISIMGRTMGALGNLTFVLCIIIFIFAVMGMQLFGKNYIDHKDRFPDHELPRWN
para            ----------------------------------------H------K-GD-----
ESPRO (d)       ----------------------------------------NV---P-KD-----
BICHON          ----------------------------------------NV---P-KD-----
GARA            ----------------------------------------NV---P-KD-----
CHANG           ----------------------------------------NV---P-KD-----
PERM (d)        ----------------------------------------NV---P-KD-----
S-LAB           ----------------------------------------NV---P-KD-----

                968
Musca           FTDFMHSFMIVFRVLCGEWIESMWDCMYVGDVSCIPFFLATVVIGNL  VVLNLFLALL
para            ----------------------------------------  ----------
ESPRO (d)       -------------------------L----------------L/F----------
BICHON          -------------------------L----------------L  ----------
GARA            -------------------------L----------------L/F----------
CHANG           -------------------------L----------------S/L----------
PERM (d)        -------------------------L----------------F  ----------
S-LAB           -------------------------L----------------L  ----------
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Figure 3. Comparison of the amino acid sequences for the *Culex* strains with the corresponding sequences for the *Drosophila para* gene and its ortholog in *Musca domestica*. (–) indicates identity among all the sequences. The only amino acid change found among the *Culex* strains is highlighted. The numbers relate to the positions in the housefly sequence.

sequence[34] confirmed that we had indeed amplified the *para*-homologous sequence in mosquitoes rather than a DSC1-homologue[35] (55% observed similarity). The three clones obtained from the susceptible strain produced two partially divergent sequences as did the two clones from the resistant strain (Fig 2A). Moreover, each of the two variants from one strain had its almost identical sequence counterpart in the other strain (Fig 2A). This intra-individual polymorphism was not uniformly distributed but concentrated on the 5′ side of a position that corresponds to an intron splicing site in *Drosophila*. In fact, after this site, no heterogeneity was found within the insects (Fig 2A). The presence of two distinct mRNAs within the two strains could be a result of a heterozygous condition, or more likely the consequence of alternative splicing. Such a splicing affecting the region upstream of the intron splicing site mentioned above has been described in *Drosophila*.[34,36] The two exons (c and d) include two non-silent changes in addition to several silent ones[34] and these changes are also conserved in *Culex* mosquitoes. Evolutionary relationships between the mosquito sequences and *Drosophila* exons (Fig 2B) clearly demonstrate the orthology between the two alternative mosquito sequences and exons c and d from *Drosophila*. Thus we propose that these two alternative exons be named c and d and hypothesize the presence of an intron in the same position as in *Drosophila*. The conservation of the same alternative splicing in *Drosophila* and *Culex* points to some important role of the alternative exons probably related to some switches during development, as seen in *Drosophila*.[36] Further studies are needed to address this issue specifically.

3.3 Analysis of the molecular basis of *kdr* in two *Culex* strains

As already mentioned, no variation within S-LAB and PERM strains was found after the position where intron 1 in *Drosophila* splices out. Therefore a single sequence is shown for each strain after that point in Fig 2A. The sequences found in the two strains are identical (including the region of alternative splicing) except for one codon: the non-silent replacement of TTA (leucine) present in the susceptible strain by TTT (phenylalanine) in the resistant strain. Thus the amino acid replacement found in the PERM strain is the same as that observed in *kdr*-resistant strains of species of four insect orders (see Section 1)

3.4 Molecular analysis of other *Culex* strains

To investigate whether this mechanism was general in *Culex pipiens*, we designed specific primers to directly sequence the PCR products from various mosquito strains and/or species without the need of cloning steps. In designing these primers, we decided to focus on one of the two alternative exons discussed above, and chose exon d since it is apparently the more common in *Drosophila* adults[36] and the only type found at present in *M domestica*.[11] Four primers were thus designed: Dipd1 and Dip2 for amplification, and Dip3 and Dip4 for direct sequencing (see Fig 2). We introduced some degeneracy so that these primers could be used for other insect species (such as *A gambiae*),[14] in considering the exon d sequence of *Culex* (presented here) and *Drosophila*[34] and the *M domestica* sequence.[11] We amplified the IIS4–IIS6 region of the voltage-dependent sodium channel using RNA extracted from single adults from the previously assayed strains of *Culex*. As a control we included the two strains that had already been cloned and sequenced. Unambiguous sequences were obtained for six individuals of the six strains and in all of them the exon d sequence was the only one present at the 5′ end of the analysed region (Fig 3) which indicated that our primers do in fact select for this sequence.

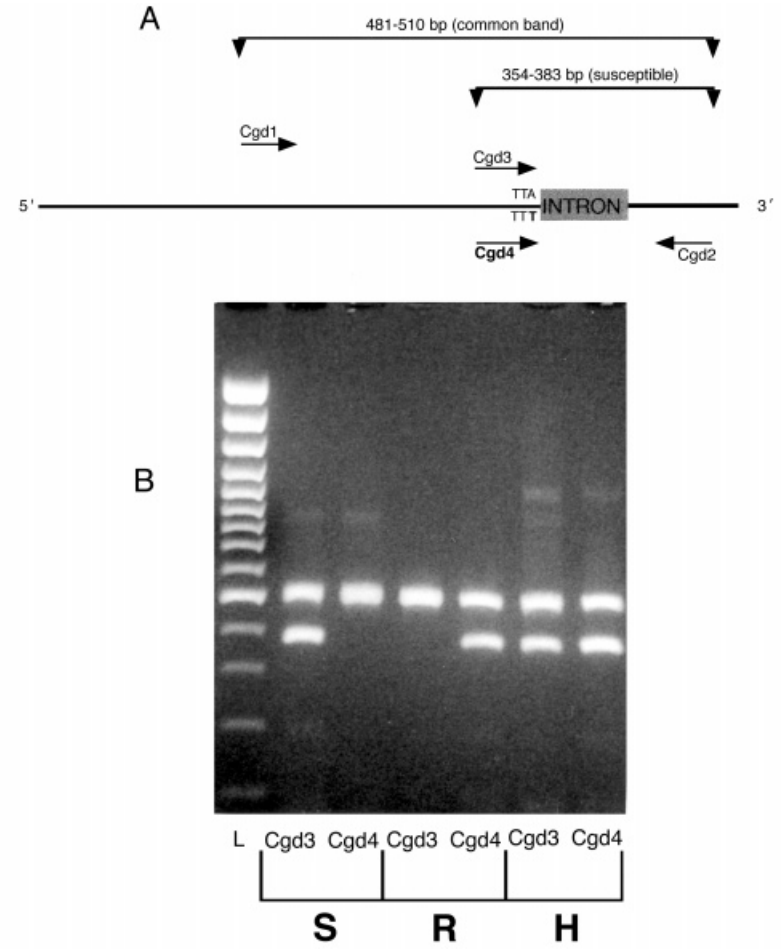

Figure 4. The diagnostic test for *kdr* in *Culex pipiens*. A: Schematic drawing of the double PCR test including the locations of the primers, the expected fragments and the position of intron 2. B: Visualisation of the fragments obtained by the test performed on genomic DNA extracted from a susceptible mosquito (S), a resistant one (R), and an heterozygote (H). Lane L: 100 bp ladder. The Cgd3 lanes correspond to PCR experiments done with Cgd1, Cgd2, and Cgd3 primers. Cgd4 lanes correspond to experiments where Cgd3 has been replaced by Cgd4 in the PCR mixture.

Sequences obtained for S-LAB and PERM were identical to those previously determined. The six nucleotide sequences were perfectly identical throughout the analysed region except at the codon position where the Leu to Phe change occurs. This triplet was found to be TTA (Leu, six over six sequences analysed) in both S-LAB and BICHON characterised as homozygous susceptible to pyrethroids by toxicological studies. The six PERM mosquitoes (homozygous for *kdr* resistance) had the codon TTT (Phe). In GARA, three individuals were homozygous for TTT, one was homozygous for TTA, and two were heterozygous TTT/TTA. In ESPRO, five individuals had TTA and one was heterozygous TTT/TTA. Finally, four individuals of the CHANG strain were homozygous for TCA which codes for a serine, one was homozygous for TTA, and the remaining individual was heterozygous TCA/TTA.

Interestingly, among the strains we assayed, CHANG had a unique profile with a low resistance to pyrethroids combined with a high resistance to DDT, which was interpreted (see above) in terms of differential modifications in the sodium channel gene. Thus we may hypothesise that, if replacing the otherwise conserved leucine residue[37] corresponding to the amino acid position 1014 in the housefly sodium channel gene sequence[11] is important for acquiring resistance to both types of insecticide, the nature of the substitution is likely to affect the relative resistance ratio to pyrethroids and DDT. However it is possible that only a limited number of amino acids may replace the conserved leucine without compromising the function of the sodium channel protein. In fact, after this report, only three different amino acids have been found in pyrethroid-resistant insects replacing the leucine present in susceptible animals: phenylalanine has been found in *M domestica*,[11] *B germanica*,[12,13] *A gambiae*,[14] *P xylostella*,[15] *M persicae*[16] and *C pipiens* (this report); histidine was found in *H virescens*,[17] and we now report the presence of a serine in mosquitoes collected in China. In this respect, it would be fruitful to introduce these alternative amino acids in the housefly sodium channel gene and compare by in-vitro expression the response of the differently modified channels to several pyrethroids and DDT as has been already done for the Leu to Phe substitution.[19]

As previously noted, no mutation similar to the one involved in super-*kdr* resistance in *M domestica* was detected in any strain.

3.5 Design of a diagnostic test for *kdr* in *Culex* pipiens

A quick diagnostic test for the presence of the *kdr* mutation in *Culex* would allow its monitoring in natural populations and thus permit improved control through better design of insecticide use. Knowing the

Table 3. *kdr* genotypes in single mosquitoes of various *Culex pipiens* strains

Strain	*S*[a]	*SR*[a]	*R*[a]	*Total*
S-LAB	10	0	0	10
BICHON	10	0	0	10
PERM	0	0	10	10
ESPRO	6	45	1	52
GARA	38	17	8	63
GARA survivors[b]	16	30	12	58

[a] S designates the presence of the TTA (Leu) codon and R the presence of the TTT (Phe) codon. Note that all tested mosquitoes were positive for the TTA or the TTT codon.

[b] Survivors of an exposure to 0.1 mg litre^{-1} of permethrin which induced a mortality of 83%.

mutation(s) responsible for *kdr* resistance allows the design of primers which will specifically amplify each of the characterised alleles. Since a good diagnostic test should be simple and rapid, RT-PCR techniques should be avoided, and a DNA-based molecular diagnostic test used. Designing such a test to identify the *kdr* mutation is complicated by the presence of an intron 4 bp downstream from the mutation (Fig 2A). In *M domestica* and *A gambiae*, this intron is highly conserved and a single PCR reaction carried out on total genomic DNA from single individuals allows the detection of the *kdr* mutation in homozygous or heterozygous insects.[11,14] A similar approach was not possible for *Culex*, since sequencing intron 2 in several individuals from the various strains used in this study revealed a high degree of polymorphism in both sequence and length (from 303 to 332 bp). Therefore, we set up a test using two PCR reactions for each individual to be diagnosed[38] and decided to concentrate only on the *kdr* mutation observed in Mediterranean countries and North America (e g phenylalanine). The two reactions were exactly the same except that one contained a sense-specific primer ending with the 'susceptible codon' (TTA) and the other contained a sense-specific primer ending with the '*kdr* codon' (TTT). Two additional common primers were included in each reaction: one antisense primer based on the sequence immediately downstream from the intron and one sense primer far upstream. The positions of the specific primers are shown in Fig 4. Each reaction yielded a common band that varied in size from 481 to 510 bp in different individuals as a result of the disparity of the length of intron 2. This band was used as an internal control for the PCR reaction. The presence of one additional band of 354 to 383 bp only occurred in the PCR reaction containing the corresponding resistance-associated specific primer (either the *kdr* specific or the susceptible one). The presence of a second band in both PCR products would be indicative of the heterozygous condition (Fig 4). The reliability of this test to detect the presence of leucine or phenylalanine codons is based on the fact that all individuals sequenced so far use the triplets TTA and TTT respectively to encode the leucine and phenylalanine present at the *kdr* position. The fact that the same codons are used by *Culex* individuals from widely separated geographical origins and even by individuals from different forms of the *Culex* complex (ie *C pipiens* and *C quinquefasciatus*) provides some degree of generality to the use of this test.

Single mosquitoes of our *C pipiens* strains were investigated using this diagnostic test. Results are summarized in Table 3. As expected, all mosquitoes from the S-LAB and BICHON strains were homozygous for the TTA codon (named S), and all mosquitoes of the PERM strain were homozygous for the TTT codon (named R). Two batches of ESPRO mosquitoes separated by five to eight generations were investigated and gave results which were not significantly different ($P = 0.56$). On a total of 52 insects tested, one was R, 45 SR and six S. Thus, knowing that *kdr* resistance is recessive, few insects should survive a permethrin exposure which should kill susceptible phenotypes (eg 0.1 mg litre^{-1}), if no other mechanism than *kdr* is present in the ESPRO strain. In fact all ESPRO larvae (560 tested) were killed by a 24-h exposure to this permethrin dose, which was not significantly different from expectations ($P = 0.12$). Two batches of GARA were also examined and were not significantly different from each other ($P = 0.76$). Eight were R, 17 SR and 38 S (Table 3). Exposure to 0.1 mg litre^{-1} permethrin killed 83% on a sample of 560 GARA larvae and the *kdr* mutation was examined among the survivors. The number of R individuals increased (from 13% to 21%) but this was not significant ($P = 0.017$). The large number of S (27%) and SR (52%) individuals confirmed that *kdr* is not the only resistance mechanism present in that strain, as already shown by the bioassays.

Hence, the results obtained by the molecular diagnostic test are in good agreement with the bioassay data. Such a PCR-based test may constitute a valuable tool to evaluate the importance of *kd* resistance in natural populations of *C pipiens* as well as of other pests of major importance.

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